Molecular foundations of prion strain diversity

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Introduction

Prion diseases are a group of fatal neurodegenerative disorders caused by prions. These unique infectious agents are not known to contain any nucleic acids and appear to be composed primarily of a misfolded form of the prion protein PrP, which is encoded by the prion protein gene *PRNP* [1,2]. Histologically, prion diseases show vacuolation, astrogliosis, neuroinflammation and deposition of PrP^{Sc}, a misfolded, beta-sheet–rich counterpart of the cellular prion protein PrP^{C}, a glycosylphosphatidylinositol-anchored (GPI-anchored) protein that is abundantly expressed in the brain. Prions propagate through the templated misfolding of PrP^{C} to infectious prion aggregates [3].

Most cases of human prion disease are sporadic (sporadic Creutzfeldt–Jakob disease, sCJD), with no known cause. Inherited *PRNP* mutations cause the genetic prion diseases familial Creutzfeldt–Jakob disease, Gerstmann–Sträussler–Scheinker syndrome (GSS) and fatal familial insomnia. Prion diseases acquired via infection include variant CJD and kuru, where prions are acquired via dietary routes, and iatrogenic CJD, caused by accidental inoculation through medical procedures [4].

Although they are caused by a misfolded form of one single protein, prion diseases of animals and humans are strikingly heterogenous and are associated with a wide range of clinicopathological variants with different symptoms, incubation periods, rates of disease progression, histology, PrP^{Sc} deposit morphologies and anatomical distributions of disease lesions [5–7].

Given that prions are not known to contain nucleic acids, these properties must be encoded in the higher-order aggregate structure. Indeed, prions exist in distinct conformational variants, known as strains, whose properties can be serially transmitted and reproduced in animal models of disease and also in human infection [6,8,9]. Furthermore, human sporadic prion disease strains can also be reproduced by the *in vitro* prion amplification technique protein misfolding cyclic amplification (PMCA), where prions are propagated in the presence of brain homogenate [10].

Individual prion strains produce PrP^{Sc} with distinct biochemical properties, such as the degree of electrophoretic mobility [11], susceptibility to protease digestion, immunoreactivity to antibodies targeting conformational PrP epitopes [12], glycosylation patterns [13] and stability on treatment with chaotropic salts [14] or heat [15].

Prion strains cause pathology with specific anatomical distributions. A study of sCJD with diffusion-weighted magnetic resonance imaging showed that the disease primary site and ordering of lesion propagation were specific to sCJD histotypes, which are caused by distinct strains. For instance, in the most common histotype, MM1, lesions spread from the neocortex to subcortical regions, with the opposite pattern seen in the histotype VV2 (see Figure 1) [16**]. The observation that many

self-templating prion strains with defined properties exist leads to the question of how their structure was formed in the original prion seed and how the strain-coding information is transmitted during prion replication. Here, we review how recent histological, genetic and biochemical studies are unravelling the molecular origins of prion strain diversity and discuss how these advances are guiding the development of innovative anti-prion therapeutics.

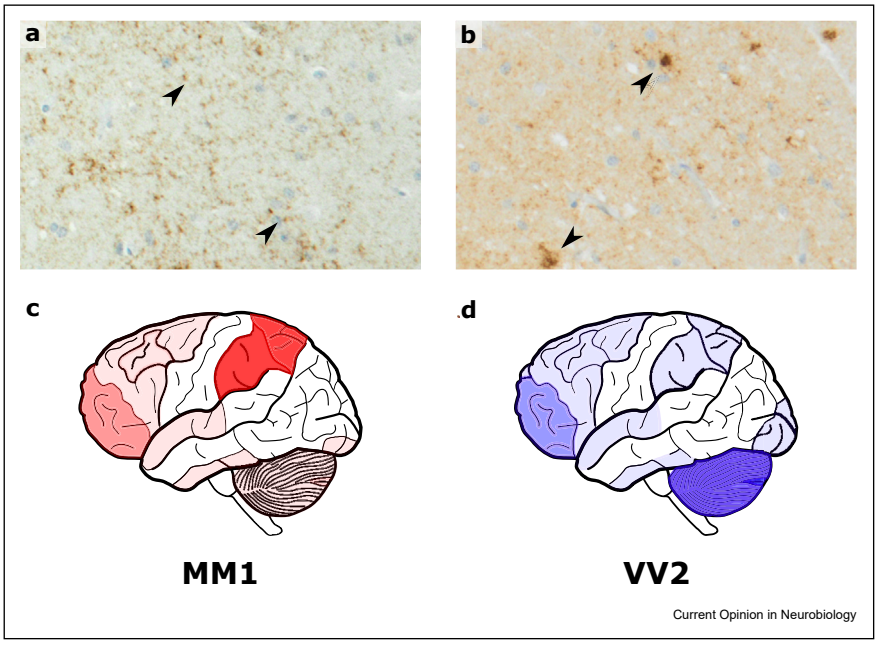
Determinants of strain properties

Prion protein primary structure

The properties of de novo prion seeds are influenced by the primary amino acid sequence of PrP. For instance, a geographically variable polymorphism in codon 129 of *PRNP*, which codes for methionine (M) or valine (V), influences biochemical features of prions when they emerge in the primary host, and these properties can be transmitted with infection [17–19]. This polymorphism also influences the risk of spontaneously developing prion disease [20], age at onset of genetic disease [21,22], clinical and histological features of disease [7] and the incubation period after exposure to prions [23]. In line with the last observation, the overall PrP sequence determines species barriers; while bank voles can be readily infected with strains originating in various species and bank vole PrP is susceptible to conversion by several human prion strains [24,25], dogs are resistant to prion infection, probably thanks to the presence of a single negatively charged amino acid (aspartic or glutamic acid) in position 163 of canine PrP, a residue not found in other species [26]. Mutations that alter the amino acid sequence of PrP can cause hereditary prion disease. Around 50–60 variants have been described, including missense mutations, truncations [27] and insertions into the octapeptide repeat region of PrP [28,29], a repetitive domain close to the PrP N-terminus likely to be a mediator of prion toxicity [30]. Full penetrance has been established for four missense mutations, which also demonstrably cause the formation of infectious prions, as shown by transmissibility experiments where rodents and non-human primates were inoculated with material obtained from diseased brains of mutation carriers; furthermore, in mice, homologous *Prnp* mutations cause spontaneous disease [31–34]. Individuals with sporadic prion disease are not known to carry any of these pathogenic mutations in the germline.

PRNP polymorphisms can also be protective. A study of persons who were exposed to kuru via cannibalistic funerary practices, but did not develop the disease, lead to the discovery of G127V, a resistance factor whose

Figure 1



Prion strains cause different disease phenotypes. PrP^{sc} aggregate morphology and anatomical spreading patterns (based on the study by Pascuzzo et al. [16**]) are shown in the CJD histotypes MM1 and VV2. MM1 is the main CJD subtype found in individuals homozygous for methionine at *PRNP* codon 129, whereas VV2 is the main type in valine homozygous individuals. Dark colour indicates the 'epicentre' of pathology, with lighter coloured regions affected later in the disease course. **(a)** Small punctate synaptic PrP^{sc} deposits in MM1, cerebellar cortex. **(b)** Plaque-like PrP^{sc} deposits in VV2, cerebellar cortex. **(c)** In MM1, diffusion-weighted imaging showed that pathology most often began in the precuneus and parietal lobe, followed by spread to the frontal and cingulate lobes, with the cerebellum and thalamus affected at late stages. **(d)** In VV2, the cerebellum is usually affected early in the disease, followed by spread to the basal ganglia and thalamus, insular cortex, frontal lobe and parietal lobe, with the occipital cortex affected at a late stage.

emergence was favoured by the selective pressure of the kuru epidemic [35]. Overall, these observations highlight how substituting a single amino acid in PrP can drastically influence the formation and propagation of prion disease, with effects ranging from protective to inexorably fatal. Several genome-wide association studies have attempted to find variants in other genes that influence the risk of developing prion disease, but the rarity of prion disease poses a significant challenge to these efforts. Apart from variants in *PRNP*, the search for associations has been mostly elusive, but weak associations with variants in *STX*6 and *GAL3ST*1 were reported recently. Risk single nucleotide polymorphisms (SNPs) in *STX6* are also shared with progressive supranuclear palsy, possibly indicative of common causal mechanisms [36**].

Aggregate biophysics

The pathogenicity of prions is associated with certain biophysical properties which lead to the formation of aggregates with characteristic morphologies. Mouse-adapted prions that form smaller, diffuse, subfibrillar aggregates and are unstable under denaturing conditions are particularly pathogenic and more likely to enter the central nervous system through peripheral nerves, whereas plaque-forming prions are likely to be less pathogenic [37]. Indeed, although both subfibrillar and fibrillar strains can be taken up by neurons by macropinocytosis and transported through axons to the cell body, only subfibrillar strains were efficiently transported to the brain. Sonicating fibrillar strains significantly increased solubility and disease attack rate on peripheral inoculation, showing that smaller PrP species are more pathogenic and that promoting fibril stability and assembly could slow prion propagation [38]. This makes sense mechanistically because the prion is defined as the minimal infectious unit, and it logically consists of one single molecular aggregate—whatever its size. Hence, taking a fibril and fragmenting it into a large number of smaller aggregates is necessarily bound to increase the number of infectious particles even if the total amount of prion protein remains unchanged. In humans, small, non-fibrillar prions are also likely to be particularly infectious. Purified protease-resistant GSS aggregates containing PrP amino acids ~90–150 were highly infectious and capable of causing disease on their own, suggesting that this part of the protein is a minimal component required for prion formation [39*].

A potentially protective prion strain has also been described. Anti-prions are self-replicating but innocuous PrP aggregates which can compete with toxic prions for their substrate PrP^{C}, resulting in its depletion, inhibiting the spread of toxic prions. When given as a prophylactic treatment in hamsters, anti-prions significantly delayed the onset of clinical symptoms after inoculation with a highly pathogenic prion strain. These findings indicate that prion replication and toxicity are uncoupled and provide a remarkable example of the phenotypic diversity of prion strains [40**].

Cofactors of prion replication

Prions typically form diffuse, synaptic or punctate deposits, whereas the formation of amyloid plaques composed of PrP is relatively rare and typically associated with a longer disease duration; however, certain strains preferentially form plaques when inoculated into wild-type mice [41*]. Therefore, genetic modifications that alter the pattern of prion deposition could be of particular interest. Whether prion strains form diffuse deposits or plaques has been proposed to be influenced by cofactors, particularly by glycosyl residues of PrP, phospholipids and polyanionic compounds such as RNA or extracellular matrix (ECM) components [42]. In particular, glycans stabilise PrP and can inhibit fibril nucleation [43,44].

A recent study explored the effect of the ECM component heparan sulphate (HS) by inoculating prions into mice which produce shortened HS chains. Prolonged survival and redistribution of PrP plaques from brain parenchyma to blood vessels were seen after infection with fibrillar, plaque-forming prions. Plaques consisted of extracellularly assembled, protease-cleaved, GPI-anchorless PrP and were also enriched in HS. Similarly, in sCJD and GSS, plaque-forming prions had the highest amounts of bound HS, whereas lower levels were seen in cases with diffuse deposits. Given that HS deficiency prolonged survival, HS was found to be a disease-modifying cofactor of prion replication that accelerates prion disease progression [45*].

A similar study investigated the significance of PrP glycosylation using 1. subfibrillar prions, 2. plaque-forming prions and 3. prions consisting of GPI-anchored PrP, which were inoculated into mice with different PrP glycosylation profiles. Expression of underglycosylated PrP resulted in prions that are usually subfibrillar forming plaques, which consisted of PrP cleaved by the protease ADAM10 and HS. The opposite effect was seen in mice producing triglycosylated PrP: here, challenge with plaque-forming prions leads to accelerated disease with deposition of diffuse, subfibrillar aggregates. GPI-anchored prions recruited unglycosylated, GPI-anchored PrP and aggregated to large plaques. However, no difference was seen after infection with the third strain consisting of unmodified, GPI-anchored PrP, which formed diffuse, HS-deficient deposits irrespective of altered glycosylation. These findings highlight how glycans, protease cleavage and GPI anchors shape prion disease phenotypes, with a strong influence on aggregate morphology, disease progression and interaction with polyanionic HS being evident. In particular, PrP plaques appeared to be

preferentially formed by underglycosylated PrP, whereas addition of glycosyl residues favoured the deposition of subfibrillar aggregates [41*].

In another study investigating the influence of cofactors on pathogenicity, a protein-only PrP^{Sc} preparation produced from recombinant PrP by the prion-amplification technique PMCA failed to induce disease after inoculation into bank voles, which are highly susceptible to infection by various prion strains. However, if the same protein-only PrP^{Sc} was seeded into a PMCA reaction containing brain homogenate, potently infectious PrP^{Sc} was produced, indicating that native PrP (not recombinant PrP produced in bacteria) and additional cofactors present within brain homogenate were required for *in vitro* production of highly pathogenic prions [46].

Using PMCA, it was also found that prion strains replicated preferentially in the presence of PrP glycoforms and cofactor molecules that were found in the host species where the prions originated. Given that PrP expression, PrP glycosylation [47], membrane lipids and ECM components vary throughout the brain, strain-specific patterns of neurotropism probably depend on the presence of PrP glycoforms and cofactor molecules in different parts of the brain, that is, prions preferentially propagate in brain regions that provide the substrates required by an individual prion strain for efficient conversion [48*].

Antemortem diagnosis of sCJD is now commonly made with real-time quaking-induced conversion (RT-QuIC) assays, which can detect prions in patients' cerebrospinal fluid with very high sensitivity [49,50]. In RT-QuIC assays, the presence of PrP^{Sc} in the analyte provokes misfolding of PrP^{C} through cycles of elongation and fragmentation, generating aggregates that can be stained with the fluorescent dye thioflavin T. It is important to understand that the original intuition of breaking aggregates and letting them regrow cyclically, which was implemented in PMCA, predates RT-QuIC by more than a decade. It was Claudio Soto who demonstrated, in a landmark experiment, that this was possible [24]. While RT-QuIC improved technically on the Soto methodology by substituting sonication with quaking, it was an evolutionary refinement rather than a revolutionary invention. As mentioned, PMCA products are demonstrably infectious. To investigate whether RT-QuIC products are pathogenic prions or an innocuous form of aggregated PrP, RT-QuIC products were inoculated into the brains of mice overexpressing human PrP, where they failed to induce clinically apparent disease. However, histological workup of the inoculated brains revealed the presence of abnormal cortical PrP deposits and vacuolation, but no evidence of gliosis was found. RT-QuIC seeding activity was also detected in the inoculated brains. These results indicate that RT-QuIC products can provoke PrP aggregation and limited cellular pathology *in vivo* but that they represent a species of PrP that is distinct from pathogenic prions [51*]. Nevertheless, the finding that a limited degree of pathology was present is of note and provides further evidence that PrP aggregates encompass a spectrum of many different species, whose degree of pathogenicity ranges from innocuous to highly infectious.

Taken together, these studies of PrP post-translational modification provide compelling evidence for a critical role of PrP glycosylation and of cofactors found in brain homogenate (possibly lipids and ECM components such as HS) in prion propagation, particularly in the formation of infectious aggregates. Furthermore, the spatial distribution of PrP post-translational modifications and of cofactor molecules in the brain might explain why individual strains preferentially seed pathology in certain brain regions.

Structural and biophysical studies

Recent studies have provided unprecedented insight into the molecular structure of prion fibrils. Using cryo-electron microscopy, a denaturant- and protease-resistant prion fibril formed from an unglycosylated fragment of human PrP was shown to contain two intertwined protofilaments with screw symmetry linked by a hydrophobic interface. Based on this structure, certain familial prion disease variants were predicted to form closely related polymorphs and a protective amino acid substitution was predicted to form steric clashes, explaining why it might disrupt prion fibril formation [52**]. A similar structural study in preprint of scrapie prions carrying post-translational modifications demonstrated how glycosyl residues markedly alter fibril structure. Moreover, GPI anchors might cause fibril–membrane interactions that are likely to be highly relevant to prion propagation and pathology [53]. The findings in these structural studies could help explain how prion strain properties are shaped by the PrP amino acid sequence and post-translational modifications, providing a molecular basis for strain diversity.

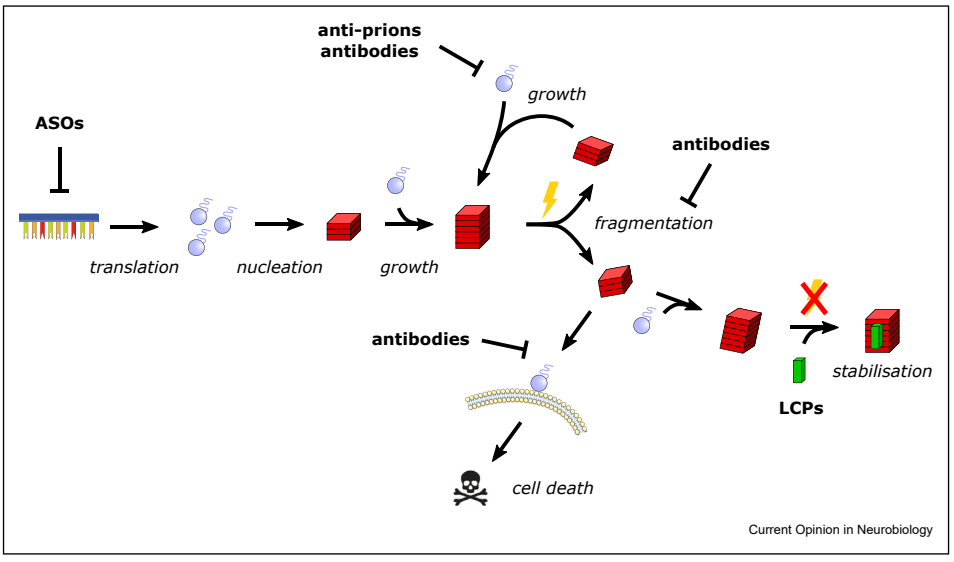
Novel biophysical and microfluidic diffusional sizing methods have enabled the study of prion propagation kinetics both *in vitro* and *in vivo*. In a recent study, such methods were used to formulate a mathematical framework of prion aggregation kinetics. *In vivo* prion aggregation kinetics of prions were consistent with *in vitro* findings and demonstrated that fragmentation was the dominant mode of prion fibril replication *in vivo* [54*].

Novel approaches to prion genetics

Somatic mutations

The rarity of sporadic prion disease renders it very challenging to use population genetics to uncover novel risk loci. Alternative approaches are therefore needed.

Figure 2



Mechanisms of action of anti-prion therapeutics. RNA coding for PrP is translated, forming PrP^C (blue molecules). At the onset of prion pathology, PrP^C forms a primary prion seed composed of misfolded monomers (red blocks). Aggregates grow by recruiting and misfolding further PrP^C monomers. Prion aggregates fragment (lightning) into smaller daughter prions, which continue to replicate by a cycle of elongation and fragmentation. Prions interact with cell surface PrP^C, initiating toxic signalling pathways. Antisense oligonucleotides bind RNA and cause it to be degraded, inhibiting expression of PrP^C. Anti-prions are non-toxic prion strains which deplete PrP^C, preventing its recruitment into toxic prions. Therapeutic antibodies bind PrP^C, with several beneficial effects: prevention of prions from binding PrP^C, decreased protein misfolding and inhibition of toxic signalling. Therapeutic antibodies and luminescent conjugated polythiophenes (LCPs) stabilise aggregates, preventing fragmentation into daughter prions.

In recent years, several remarkable studies have shown how the human brain is a genomic mosaic, with individual cells harbouring clonal or private mutations that were acquired as a consequence of mutagenic processes, some of them potentially altering the function of affected genes [55,56]. This genomic diversity raises the possibility that somatic mutations in key disease-related genes could contribute to neurodegeneration, including to the pathogenesis of prion disease, and several recent studies have begun to investigate this possibility.

With ageing and in Alzheimer's disease, a number of putatively pathogenic mutations were detected in pathways implicated in hyperphosphorylation of tau, suggesting that they might have contributed to Alzheimer's disease pathology [57*]. A similar study of semantic dementia revealed individuals with putatively pathogenic mosaic mutations in *TARDBP* (with a mutant allele fraction of 1−3%), one of the main genes implicated in the pathogenesis of this neurodegenerative disease [58]. A study of the synucleinopathies Parkinson's disease and multiple system atrophy found that nigral dopaminergic neurons harboured somatic *SNCA* (α-synuclein) copy number gains compared with controls, suggesting that supernumerary *SNCA* copies, possibly leading to overexpression of α-synuclein, might be a significant pathogenetic risk factor [59]. Indeed, germline locus triplication of *SNCA* is a long-known cause of hereditary Parkinson's disease [60].

Somatic mutations were already proposed to lie at the origin of sporadic prion disease around 30 years ago [61], but there are no reports on large-scale searches for *PRNP* mutations in prion disease samples. Although biosafety hazards and the limited availability of patient samples render it difficult to sequence DNA from the prion-contaminated brain, targeted sequencing of *PRNP* or whole genome sequencing of the somatic genome could potentially uncover known pathogenic variants or novel genetic risk factors.

Screens

Genetic screens might also identify crucial genes hitherto undetected by population genetics. An arrayed whole-transcriptome RNA interference screen in preprint identified a number of genes regulating PrP^C biosynthesis, with the RNA-binding post-transcriptional repressor Pumilio-1 emerging as a potent regulator causing degradation of *PRNP* mRNA [62]. Similar screens for genes regulating prion propagation or production of potential prion co-factors could help unravel prion pathogenesis and disease heterogeneity. The 'hits' uncovered by such unbiased screens could represent genetic risk loci for sporadic prion disease, potentially containing disease-modifying variants in affected

patients. In a different screen using a short-hairpin RNA library targeting 133 genes related to protein homeostasis, several members of the Hsp70 family of chaperones and related proteins were identified as regulators of PrP^{C} expression. A molecule inhibiting the Hsp70 family reduced PrP^{C} levels and also PrP^{Sc} formation [63]. Although this finding could hold promise for the development of small-molecule therapeutics, it might be necessary to find a more specific Hsp70 family member that is of relevance to prion pathophysiology, as the pervasive functions of the Hsp70 family in cell biology might make them too broad a target [64].

Therapies

Next, we discuss how recent findings in the field of prion research could inform the development of anti-prion therapeutics (see the overview in Figure 2).

Aggregate stabilisation

Given that prions replicate via fragmentation, stabilisation of aggregates with molecules that intercalate into the quaternary structure of prions could be a promising therapeutic strategy. Luminescent conjugated polymers bind prions and other amyloids [6,65], stabilise prions [66] and extend the lifespan of prion-infected mice by up to 80%, while being well-tolerated [67]. Given that these molecules are luminescent, imaging approaches could be used to track their function and distribution *in vivo*. Using similar compounds and multiphoton microscopy, longitudinal imaging of the spread of tau and amyloid was achieved *in vivo* [68**]. Similar studies of prions, with longitudinal imaging surveillance of their effects, might help identify promising drug candidates. Polymers that specifically bind prion aggregates could also be conjugated to effector molecules, such as immunological signalling proteins, for specific targeting of aggregates.

Antibodies

Anti-PrP antibodies exert differential effects depending on their targeted epitope. Antibodies binding the C-terminal globular domain of PrP are toxic and reproduce many of the features of prion disease, whereas antibodies binding the N-terminal flexible tail protect against disease, presumably by preventing prions from binding PrP^{C} and exerting toxic effects [30]. Recent data from our laboratory show that expression of PrP^{C} in neurons—but not astrocytes—is required for prion toxicity [69]; hence, preventing prions from binding neuronal PrP^{C} with suitable antibodies might be a viable strategy to prevent neurodegeneration.

Anti-PrP autoantibodies, including clones with therapeutic potential, were recently shown to be found in healthy individuals. No associations with manifest prion disease, *PRNP* mutation status or any other specific pathologies could be identified, indicating that circulating anti-PrP antibodies are likely to be well-tolerated, making them a viable therapeutic approach [70,71]. Rationally designed therapeutic anti-PrP antibodies have already shown promise in early experimental studies. A bispecific antibody which simultaneously targets both the globular and flexible tail domains of PrP reduced neurotoxicity and the formation of soluble oligomers in a brain slice model of prion disease, possibly by blocking the prion-binding site in the globular domain and stabilising the flexible tail [72]. Further studies, particularly *in vivo* treatments, are required to determine whether anti-PrP antibodies might hold promise as a neuroprotective therapy or possibly also as promoters of prion clearance. In particular, whether limited passage through the blood—brain barrier could be a hindrance to therapy remains to be elucidated.

Antisense oligonucleotides

Gene knockout of mouse *Prnp* produces a demyelinating polyneuropathy phenotype, as PrP is required for myelin maintenance in peripheral nerves [73,74], but in the central nervous system, lack of PrP expression is currently not known to produce significant effects. Consequently, depleting PrP in the brain might be a promising therapeutic strategy to treat prion disease or a prophylactic strategy to prevent *PRNP* mutations from causing outbreak of genetic prion disease (as in the anti-prion approach described previously). A promising strategy to reduce PrP expression is knockdown using antisense oligonucleotides (ASOs), single-stranded DNA fragments that specifically bind their target mRNA, leading to its degradation by RNase H1. Chronic suppression of PrP expression with ASO treatment extended survival of prion-infected mice in both prophylactic and therapeutic settings, even if given shortly before the onset of evident neuropathology [75**].

In a follow-up study, ASO treatment conferred robust dose-dependent benefits in mice infected with 5 different prion strains, with 21% RNA knockdown already extending survival. ASOs even extended survival if given after the onset of clinically evident disease, in which case they attenuated symptoms and lowered concentrations of biomarkers of neuronal injury [76]. Given that ASOs act by depleting the substrate of prion conversion, their action is independent of strain properties, meaning that they could be effective in all forms of prion disease. This is in contrast to stabilising agents such as luminescent conjugated polymers, which would probably only be effective against certain strains. Furthermore, ASOs could potentially be designed to specifically knock down mutant variants of *PRNP* and be used as a prophylactic treatment of *PRNP* mutation carriers, which might delay or even prevent the outbreak of disease. Combination therapies with ASOs and stabilising agents or antibodies might be

particularly promising. Stabilising agents would confer the additional benefit of inhibiting prion fragmentation, whereas antibodies might enhance prion clearance or prevent the toxic cascade initiated by prions on binding of PrP^{C}. Overall, combination therapies might give microglia time to clear prions, significantly prolonging survival.

Conclusion

The observation that prion strains composed of a single protein are capable of causing a diverse range of pathological phenotypes has baffled scientists for many decades. Recent advances have brought unprecedented insight into the genetic and biochemical phenomena that shape prion strain diversity. Even though prions are believed to principally consist of protein, the role of non-protein cofactors such as glycans, polyanionic macromolecules and GPI anchors in prion propagation is becoming increasingly apparent. Furthermore, structural studies are elucidating the influence of mutations and post-translational modifications on the molecular architecture of prions. Although prion propagation is increasingly understood—with aggregate fragmentation being shown to play a critical role—how prions begin their existence in the first place is still shrouded in mystery. Here, emerging fields such as the study of genomic heterogeneity and high-content genetic screens might provide further insight into the origin and propagation of prions. A deeper understanding of prion diversity is also likely to inform design of specific anti-prion therapeutics, with approaches that stabilise aggregates, target critical prion epitopes or deplete prion protein holding particular promise.

Conflict of interest statement

Nothing declared.

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** of outstanding interest

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